Identification and Characterization of Two Isozymic Forms of Arylamine N-Acetyltransferase in Syrian Hamster Skin

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Arylamine N-acetyltransferase (EC 2.3.1.5) activity was examined using skin from Syrian hamster. Two isozymes of arylamine N-acetyltransferase, designated NAT-1 and NAT-2, were detected on anion-exchange high-performance liquid chromatography analysis. Both enzyme activities had indistinguishable molecular masses (30 kDa), but differed significantly in their specificity toward the aromatic amines including serotonin, dopamine, methoxytryptamine, tryptamine, para-phenetidine, para-aminobenzoic acid, and sulphamethazine. Specifically, NAT-2 but not NAT-1 catalyzed acetylation of dopamine to N-acetyldopamine and acetylation of serotonin to form N-acetylserotonin, a direct precursor of melatonin. The two isozymes were also distinguishable based upon their sensitivity toward methotrexate inhibition (50% inhibiting dose for NAT-1 = 380 μM; NAT-2 > 2 mM). The presence of these two activities in the skin raises new questions about the physiologic role of this enzyme in general and in the skin-specific functions in particular. Key words: N-acetylserotonin/N-acetyldopamine/detoxification/melatonin. *J Invest Dermatol 101:660–665, 1993*

The skin is the largest body organ that, besides being a physical barrier, possesses a complex defense mechanism against physical, chemical, and biologic stressors [1]. Thus, it is a powerful immune organ that contains an important local component of immune response-associated cells (keratinocytes, endothelial cells, mast cells, tissue macrophages, T lymphocytes, and dendritic cells) [1]. It has endocrine functions as well, e.g., it produces vitamin D_3 [2] and PTH-like hormone [3,4], expresses proopiomelanocortin (POMC) [5], and is involved in steroid metabolism [6]. Furthermore, the skin possesses a large enzyme repertoire involved in drug and xenobiotic metabolism that maintains or modulates its chemical milieu [6].

The hamster is a rodent model with a long tradition in dermatologic research, particularly in contact sensitivity, wound healing, mast cell studies, and notably the form of the "hamster flank organ" assay in drug-permeability studies [7,8]. The approximately 2% incidence of spontaneous melanoma in hamsters makes this animal a valuable tool for the study of malignant transformation of melanocytes [9,10]. In addition, transplantable melanomas in hamsters have been used as models for pigment cell and melanoma research and to examine multidirectional interactions between cutaneous and tumor tissues [11,12]. Recent studies by Kawakubo *et al* [13] demonstrating acetyltransferase activities catalyzing N- and O-acetylation of carcinogenic arylamines and N-hydroxylamines in hamsters suggest that the skin may play a role in the metabolism of aromatic amines. This places this animal high on the list of experimental models for studies concerning cutaneous metabolism of aromatic amines and its relationship with skin physiology or pathology.

Two classes of aromatic amine N-acetyltransferase (NAT) activities have been described [14]. One of these activities, arylalkylamine NAT, catalyzes the acetylation of serotonin to N-acetylserotonin, which is the key regulatory step in the synthesis of melatonin [15,16]. Melatonin is synthesized in the pineal gland, retina, and perhaps in other peripheral sites undetected so far. It has been shown, for example, that lymphocytes can synthesize melatonin [17,18]; it also has been suggested that melatonin can be synthesized in the gastrointestinal tract [19]. Melatonin is an indoleamine with multiple regulatory functions, e.g., besides regulation of the circadian rhythm, it may act as a neurotransmitter, hormone, and immunomodulator [20]. The mammalian skin is one potential target organ for melatonin bioregulation [21].

The other class of NAT demonstrates a preference for arylamine substrates such as phenetidine and sulphamethazine; hence this class of activity is referred to as "arylamine NAT." Although this enzyme activity has been primarily studied in the liver and is thought to be involved in the detoxification of exogenous amines (for review see [22,23]), arylamine NAT activity has also been detected in the hamster skin [13]. Attenuation of this NAT activity has been associated with several disease processes such as bladder cancer [22,23]. Earlier studies done primarily in rabbits and humans indicate that there are at least two categories of this enzyme activity, i.e., polymorphic and monomorphic, which can be assessed based primarily upon substrate specificities. This acetylation polymorphism has

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Abbreviations: CoASAc, acetyl-coenzyme A; NAT, N-acetyltransferase; MTX, methotrexate; POMC, proopiomelanocortin; SMZ, sulphamethazine; WAX, weak anion-exchange.

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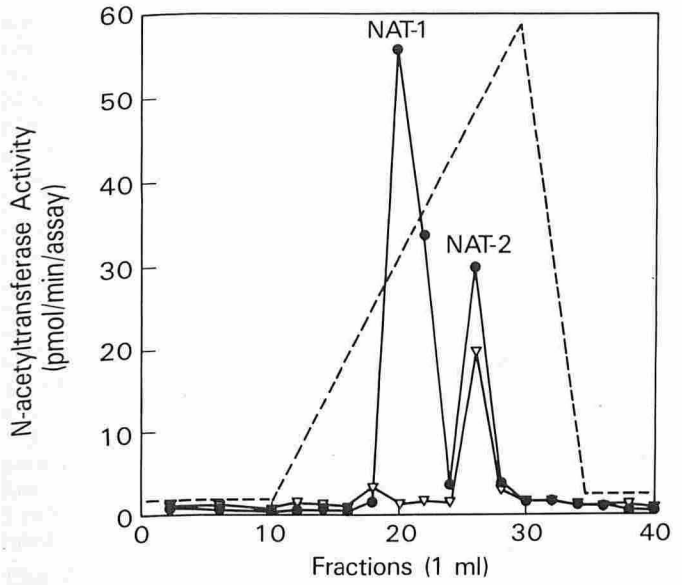


Figure 1. Arylamine NAT activity of skin supernatant as shown by anion exchange chromatography using a WAX HPLC column. A 20-ml supernatant sample of a 20% skin homogenate was loaded onto the column. The column was washed using equilibrating buffer and the enzyme activity was eluted using a linear gradient of KCl (0–1.0 M) in Tris-HCl (20 mM, pH 7.3), containing 1 mM dithiothreitol. Fractions (1 ml) were assayed using both arylamine *(circle)* and arylalkylamine *(triangle)* substrates. Two peaks of NAT activity are depicted. NAT-1 demonstrates specificity toward the arylamine substrate, whereas NAT-2 acetylates both the arylamine and arylalkylamine substrate. *Dashed line,* salt gradient.

been observed more recently in hamster populations as well, but the pattern of substrate acetylation is opposite to that observed in human and rabbit [24]. Two distinct NATs were identified from inbred hamster liver preparations in which one NAT activity was shown to be polymorphic whereas the other was classified as monomorphic [25]. The amine specificities for the two types were clearly different from those found in the case of rabbits and humans.

In the first step toward elucidation of the mechanism regulating cutaneous metabolism of aromatic amines and its potential role in skin physiology and pathology, we have identified and characterized two isozymes of arylamine NAT in hamster skin. Our results demonstrate two ionic species of NAT activity (NAT-1 and NAT-2), which, based upon substrate specificity, are classified as an arylamine NAT (NAT-1) and a mixed arylamine/arylalkylamine NAT (NAT-2). Furthermore, they differ in their spectrum of specificities as well as in other biochemical parameters.

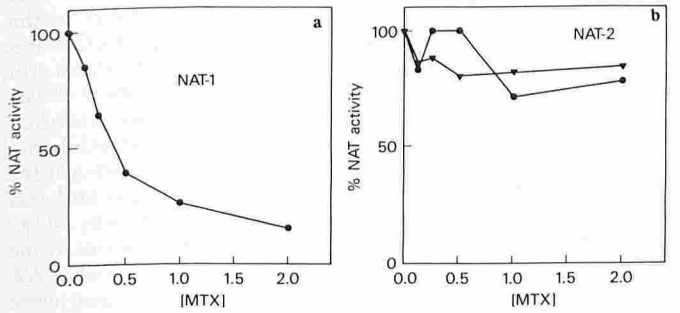
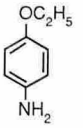
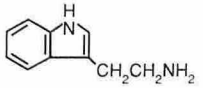
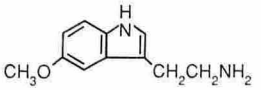
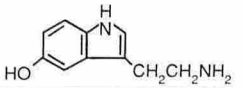


Figure 2. Concentration dependency of inhibition of NAT activity by MTX. Here the MTX ID_{50} curve following the incubation of the NAT sample, after the anion exchange procedure, with various concentrations (0–2 mM) of MTX is seen. Subsequently, NAT enzyme assays were performed using the MTX-incubated NAT samples. Only arylamine *(circle)* NAT activity was assayed in the case of the NAT-1 samples; however, for the NAT-2 samples both arylamine *(circle)* and arylalkylamine *(triangle)* NAT activities were examined. The values plotted are the mean of duplicate determinations, which were within 5% of the mean. The ID_{50} value for NAT-1 *(a)* was determined to be 380 μM, whereas NAT-2 *(b)* did not demonstrate significant sensitivity toward MTX using either amine.

Table I. Substrate Specificity of Hamster Skin to Arylamine N-acetyltransferase-2[a]

Amine Substrate	pmol/min/assay 0.1 mM	pmol/min/assay 1 mM	Amine Structure
Para-phenetidine	29.6	23.9	
Tryptamine	6.4	13.2	
Methoxytryptamine	11.0	16.6	
Serotonin	0.8	0.0	

[a] The substrate specificity of NAT-2 was evaluated using both classes of aromatic amine substrates. The arylamine substrate is para-phenetidine, and the arylalkylamine substrates are serotonin, tryptamine, and methoxytryptamine. Two substrate concentrations are used in this experiment, 0.1 mM and 1 mM, which provides a range for the amine substrates being examined. The data presented are means of duplicate determinations that were within 5% of the means. Similar substrate specificity patterns were observed in two separate experiments.

MATERIALS AND METHODS

Preparation of the Enzyme Extract Male Syrian golden hamsters *(Mesocricetus aureauts)* were purchased from Charles River, Wilmington, MA, housed in community cages in the Albany Medical College Animal Facility with 12-h light periods, and fed *ad libitum* with water and rat/mouse chow. Three- to six-month–old hamsters were used as a source for the skin. The animals were sacrificed and their hair was removed with an electrical animal clipper. All back skin was dissected at the level of subcutis, immediately transferred to liquid nitrogen, and stored at −80°C. The animal protocols were approved by the IACUC at the Albany Medical College.

A 20% homogenate was prepared in 20 mM Tris-HCl (pH 7.3) containing 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 30,000 × *g* for 2 h.

Weak Anion Exchange High-Performance Liquid Chromatography (HPLC) Weak anion exchange (WAX) chromatography was employed for the analysis of potential ionic forms of NAT activity in the crude enzyme preparations from the skin. The supernatant (approximately 20 ml) was injected into the WAX HPLC column (BioSeries, 5 cm × 7.5 mm; MacMod Analytical, Inc., Chadds Ford, PA). The equilibrating buffer was 20 mM Tris-HCl (pH 7.3) containing 1 mM dithiothreitol (flow rate, 1 ml/min). The effluent was collected on ice and later assayed for enzyme activity. The column was washed with equilibrating buffer for 10 min and the enzyme activity was eluted using a KCl gradient (0–1.0 M) in the equilibrating buffer. Fractions (1 ml) were collected and assayed for NAT activity using both para-phenetidine and tryptamine (arylamine and arylalkylamine substrates, respectively).

Size-Exclusion HPLC NAT-1 and NAT-2 following the WAX HPLC procedure were collected separately, concentrated using microconcentrators (10 k molecular weight cutoff; Amicon, Beverly, MA), and subsequently subjected to size-exclusion HPLC (TSK-G3000SW, 60 cm × 7.5 mm; Thomson Instruments, Springfield, VA). The column was equilibrated with 100 mM ammonium acetate (pH 7.3) containing 1 mM dithiothreitol. A 0.5-ml sample was injected and the elution was performed using the equilibrating buffer at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and assayed for NAT activity using both para-phenetidine and tryptamine.

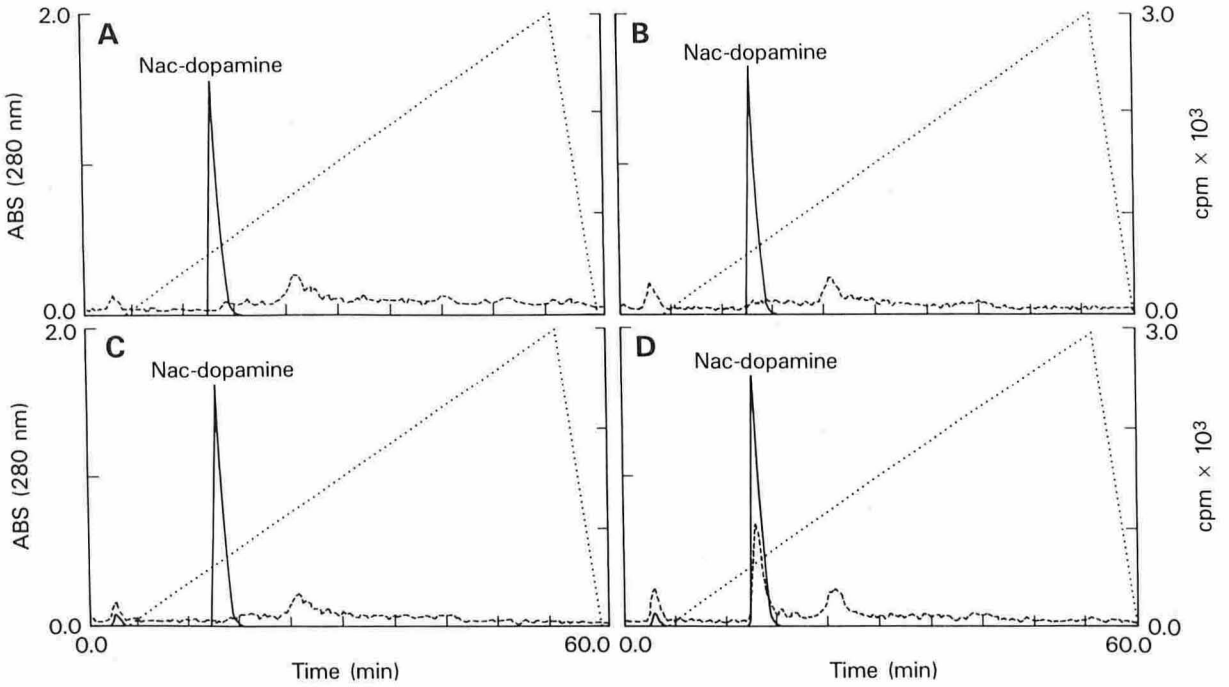


Figure 3. N-acetylation of dopamine is depicted using reverse-phase HPLC. NAT enzyme assays were performed using dopamine as the amine acceptor. *A,C*, controls (absence of enzyme) for NAT-1 *(A)* and NAT-2 *(C)*; *B,D*, chromatographic results when enzyme was included in the reaction assay for NAT-1 *(B)* and NAT-2 *(D)*. *Dotted line,* methanol gradient (0–100%); *solid line,* absorbance at 280 nm; and *dashed line,* radioactivity.

NAT Assay A modification [14] of Deguchi and Axelrod's [26] method was employed using para-phenetidine (1 mM) and tryptamine (1 mM) as the amine acceptors and [^{3}H] acetyl Coenzyme-A (CoASAc, specific activity = 20 μCi/μmol, 0.1 mM final; Amersham Corp., Arlington Heights, IL) as the acetyl donor. A 25-μl portion of the tissue sample was incubated (37°C, 30 min) with 12.5 μl [^{3}H]CoASAc and 12.5 μl of amine substrate, both prepared in 0.1 M sodium phosphate buffer (pH 7.2). The reaction was terminated by the addition of 1 ml of chloroform, the reaction tubes were vortex-mixed, and the aqueous layer was aspirated. The chloroform phase was washed twice with 0.2 ml of 1 N NaOH and a 500-μl aliquot was placed in a scintillation vial for evaporation; subsequently, radioactivity was measured using liquid scintillation counting.

When assaying with serotonin, the method described by Deguchi and Axelrod [26] was used with a minor modification. This modification involved washing the organic phase three times with 0.2-ml aliquots of NaOH (1 N). All of the samples in the substrate specificity study were processed in this manner.

Methotrexate (MTX) Inhibition of NAT Activity A 50% inhibiting dose (ID_{50}) value of methotrexate (MTX) was determined for the NAT activity following WAX HPLC analysis of the crude skin homogenate. Various concentrations of MTX (0–2 mM) were incubated (4°C, 24 h) with NAT-1 and NAT-2 independently. Following a 24-h incubation, the enzyme assay was performed using para-phenetidine and tryptamine where appropriate, specifically with NAT-2. The mode of inhibition was assessed by performing kinetic analysis on NAT-1 using 1 mM and 2 mM MTX and assaying with para-phenetidine. The mathematical analysis was performed using Sigma Plot version 4.1.

Reverse-Phase HPLC Analysis In order to determine the NAT activity for para-aminobenzoic acid (PABA) and sulfamethazine (SMZ) (both of which are arylamines), a reverse-phase HPLC system with radioisotope detection was employed. The NAT assay itself was essentially the same as described above (amine concentration was 1 mM and 0.1 mM). Following the incubation, however, the samples were processed differently. The reaction was terminated with 0.2 N $HClO_4$ (50 μl). Approximately 90 μl was injected into the reverse-phase HPLC column (VYDAC-C_{18}, 0.5 cm × 22 cm; Princeton, NJ), which was equilibrated using 1% glacial acetic acid (pH 4.0, flow rate = 1 ml/min). The elution was different depending upon the amine substrate used. For SMZ, the column was washed for 5 min using the equilibrating buffer, then a 12% isocratic elution was established using methanol (88 : 12, acetic acid : methanol). When determining PABA acetylation, a 5% isocratic elution was established with methanol (95 : 5, acetic acid : methanol) following a 5-min wash with equilibrating buffer. A Beckman 171 radioisotope detector was employed to determine the radioactivity of the sample injected (efficiency = 16%); the data presented are uncorrected. Acetylated standards were prepared by using acetic anhydride with the appropriate amine [27].

To analyze dopamine acetylation, a linear gradient using methanol was established. Acetic acid (1%, pH 3.0) was used as the equilibrating buffer and the gradient, which employed methanol, was initiated following a 5-min wash with the equilibrating buffer. The gradient continued for 55 min, until 100% methanol was achieved.

RESULTS

Demonstration of Two NAT Activities in the Hamster Skin To address the possibility of multiple NAT species in the skin, an approach similar to that taken by Hein *et al* [25] and Grant *et al* [28] was employed. Using Syrian hamster skin supernatant prepared from a 20% homogenate, the ionic characterization of the skin NAT activity was performed using anion-exchange chromatography (Fig 1). The chromatographic profile of the anion-exchange procedure demonstrated that the NAT activity was bound to the column under the described loading conditions. Essentially no enzyme activity was detected in either the effluent or wash. Alternate fractions were assayed using both classes of aromatic amine substrates (para-phenetidine and tryptamine). Two peaks of NAT activity were observed. The first peak of NAT activity, designated NAT-1, eluted approximately midway through the KCl gradient. Based upon substrate specificity, NAT-1 is characterized as an arylamine NAT. The other NAT activity peak, which eluted following NAT-1, demonstrates both arylamine and arylalkylamine acetylation; however, the predominant NAT activity is arylamine. Arylalkylamine NAT activity accounts for approximately 75% of the total NAT activity in NAT-2.

Sensitivity to MTX Inhibition is Examined To further characterize NAT-1 and NAT-2, we examined the behavior of each activity individually toward methotrexate (MTX). In previous

work, we have been able to demonstrate differential sensitivity toward MTX inhibition [29]. Another element in the strategy of employing MTX is an attempt to address the question of whether NAT-2 represents one or two separate NAT activities. NAT-1 and NAT-2 were incubated with various concentrations of MTX overnight at 4°C. The following day the various preparations were assayed for NAT activity. In the case of the NAT-2 preparations, both classes of amine substrate were employed, i.e., arylamine (para-phenetidine) and arylalkylamine (tryptamine). Figure 2 represents MTX inhibition curves from which ID_{50} values were evaluated. NAT-1 clearly demonstrates sensitivity to MTX inhibition in which the ID_{50} value is determined to be 380 μM (Fig 2*a*). In contrast, NAT-2 appears not to be affected by MTX concentrations up to 2 mM (Fig 2*b*). Equally important is the finding that both NAT activities behave in a similar manner, suggesting that NAT-2 is one ionic NAT species with broad substrate specificity.

The mode of MTX inhibition on NAT-1 was assessed using para-phenetidine as the amine substrate. A family of near-parallel lines were observed upon performing kinetic analysis using 0, 1, or 2 mM MTX (data not shown). This suggests that the mode of inhibition is uncompetitive.

The Amine Substrate Specificity for NAT-2 is Studied To further pursue the specificity of NAT-2, we conducted a substrate specificity study (Table I). Following the WAX HPLC procedure, NAT-2 samples were subjected to substrate-specificity analysis. As Table I illustrates, clearly the predominant activity is against the arylamine substrate, para-phenetidine. However, it also demonstrated significant activity toward arylalkylamines. This NAT activity appears to prefer the more hydrophobic arylalkylamine substrates, such as tryptamine and methoxytryptamine, when compared to serotonin, which is less hydrophobic.

In contrast to this finding, Fig 3 illustrates enzymatic acetylation of dopamine, which is less hydrophobic than serotonin. This property appears to be specific to NAT-2 because NAT-1 shows no acetylating activity toward dopamine. Acetylation occurs using 1 mM dopamine with little observed when 0.1 mM dopamine is employed, perhaps indicating a relatively high K_m for dopamine.

The difference in acetylation of PABA and SMZ between NAT-1 and NAT-2 is illustrated in Fig 4, which only represents data obtained using 1 mM substrate concentration. The pattern of acetylation remains the same when 0.1 mM is employed (data not shown). NAT-2 clearly demonstrates the ability to acetylate SMZ (Fig 4*b*), whereas NAT-1 does not appear to acetylate this amine (Fig 4*c*). When the substrate is PABA, both NAT-1 and NAT-2 manifest the ability to acetylate this molecule (Fig 4*e* and *f*, respectively).

Size-Exclusion HPLC Size-exclusion chromatography was employed to further purify and to obtain the approximate molecular mass of NAT-1 and NAT-2. These two NAT activities were collected separately following the WAX HPLC procedure, concentrated to 0.5 ml, and subsequently subjected to size-exclusion HPLC (Fig 5). Fractions (25–45) were collected and assayed using both para-phenetidine and tryptamine. As Fig 5 illustrates, both NAT-1 and NAT-2 have identical retention times. NAT-1 demonstrates substantial arylamine NAT activity when compared to the arylalkylamine NAT activity (Fig 5*a*). In contrast, Fig 5*b* illustrates significant arylalkylamine NAT activity co-eluting with the arylamine NAT activity peak in NAT-2. This arylalkylamine NAT activity is approximately 75% that of the arylamine NAT activity.

DISCUSSION

The results presented above demonstrate the presence of at least two isozymic forms of arylamine NAT activity in Syrian hamster skin. These two isoforms were resolved upon performing anion-exchange HPLC of the crude supernatant. These two skin NAT isoforms can be further distinguished based on their amine substrate specificity and sensitivity to inhibition by MTX.

It is of interest to note that two ionic forms of NAT activity have been detected earlier in the hamster liver [24]. Consistent with the earlier results, NAT-1, the activity eluting first on anion-exchange chromatography, was least active against sulphamethazine, one of the most commonly used drugs employed to characterize arylamine NAT activity in the liver. Earlier studies [25] have also shown that this enzyme activity (NAT-1) exhibited polymorphic variation in activity among various individuals. In contrast, in other species examined, the polymorphic forms of the enzyme were found to be highly active against sulphamethazine. It is not clear that the NAT-1 we have detected in the skin also shows polymorphic variation. The enzyme activity in the liver is thought to be involved in the detoxification of arylamine and hydrazine drugs [22,23]. Whether the enzymes in the skin also participate in a similar function remains unclear. Recent studies indicating that multiple forms of this enzyme (NAT) activity are present in other tissues, including the brain [29,30], raise the distinct possibility that they may serve additional functions related to the maintenance of normal physiologic processes.

Our results provide insights into two such potential roles for at least one of the two enzyme activities, i.e., NAT-2. We have found that NAT-2 acetylates a variety of arylalkylamines, including serotonin, with reasonable potency. This raises the possibility that this enzyme may be involved in acetylating biogenic amines either for detoxifying them or to generate biologically active products such as melatonin. Recent observations [17,18] that lymphocytes can synthesize melatonin show that melatonin synthesis is not restricted to selected tissues such as pineal and retina, as was previously thought. Based on this, it is reasonable to suggest that perhaps skin, a tissue in which melatonin is thought to be active, also can synthesize melatonin for local use.

The second property of NAT-2, acetylation of dopamine to N-acetyldopamine, is of special interest. To our knowledge this is the

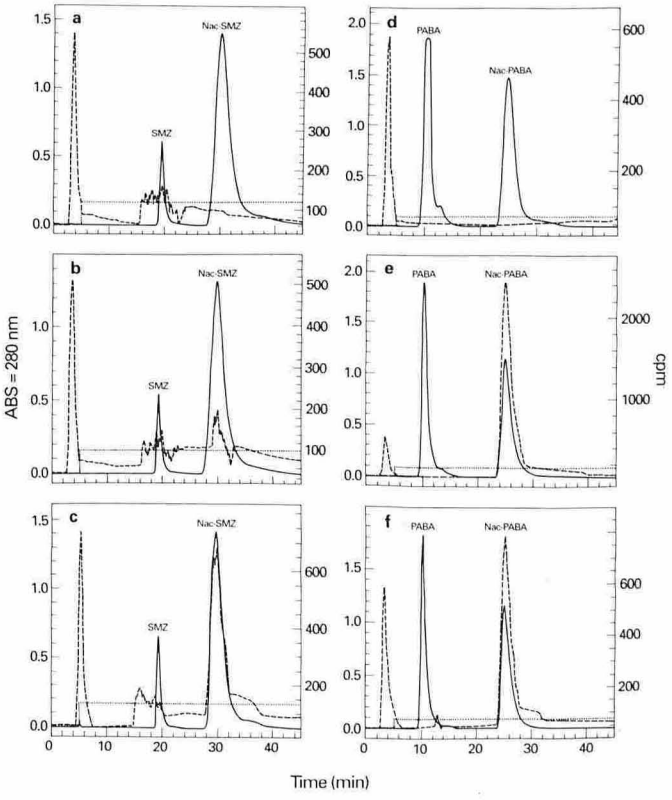
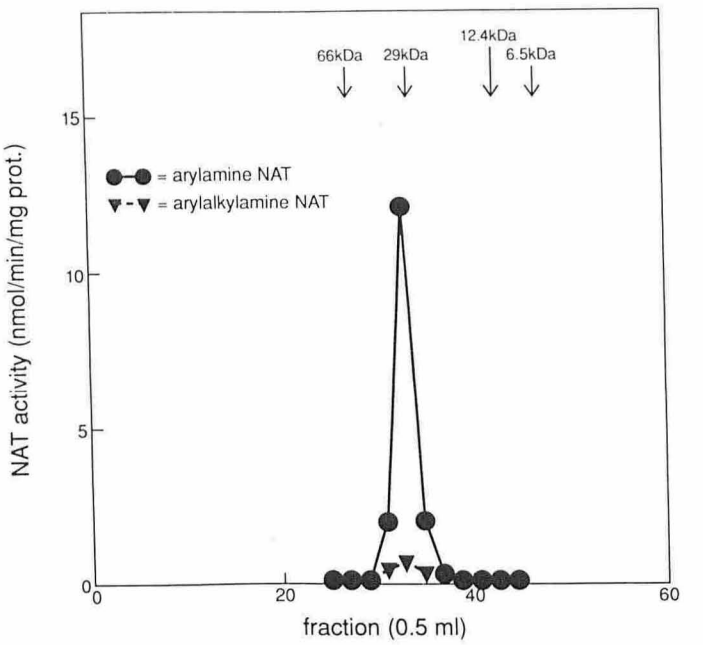


Figure 4. Reverse-phase HPLC analysis of NAT activity using PABA and SMZ. NAT-1 and NAT-2 were incubated with 1 mM SMZ (*b* and *c*, respectively) as well as with 1 mM PABA (*e* and *f*, respectively). *a,d*, assays in which phosphate buffer (pH 7.3, containing 1 mM DTT) plus SMZ *(a)* or PABA *(d)* was substituted for NAT-1 and NAT-2. *Solid lines,* absorbance at 280 nm; *dotted lines,* isocratic elution; *dashed lines,* radioactivity.



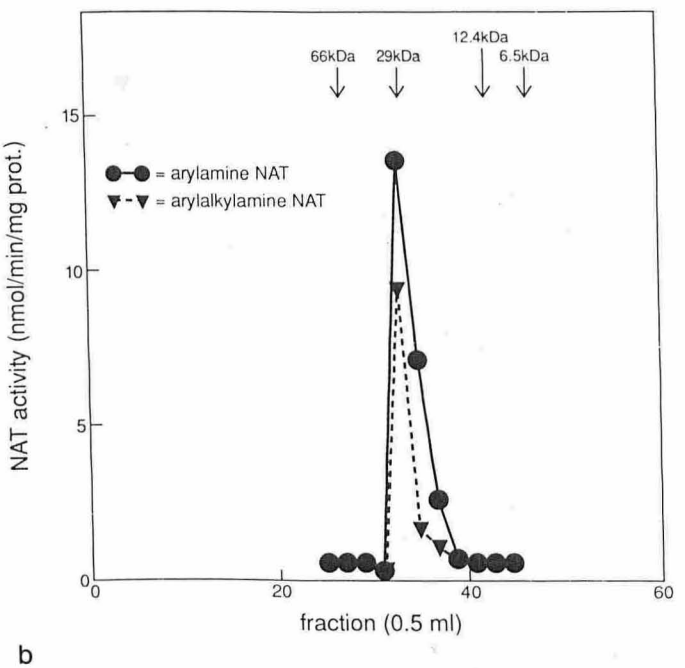


Figure 5. Size-exclusion HPLC of WAX HPLC NAT-1 *(a)* and NAT-2 *(b)* activities. Fractions were collected (0.5 ml) and assayed for both arylamine (para-phenetidine) and arylalkylamine (tryptamine) NAT activity. The molecular mass markers *(arrows)* used were bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa).

first demonstration of the enzymatic acetylation of dopamine in the mammalian species. Although the role of this enzyme remains speculative, the significance of this reaction for tissue homeostasis can be envisaged. The acetylation of dopamine may play a role in the regulation of local tissue concentration of this biogenic amine or in attenuating its bioregulatory activity, for example, on the cutaneous vascular system. Alternatively, N-acetyldopamine is itself a biologically active molecule. In this context, we note that N-acetyldopamine has been detected in the urine and kidney tissue of humans [31–33]. In the retina, dopamine is one of the main negative regulators of melatonin synthesis, e.g., it is released during exposure to light and by activating the D_2 receptors on photoreceptors, which inhibits NAT activity [34]. This raises the intriguing possibility that the acetylation of dopamine may play a role in disinhibition of arylalkylamine NAT activity, a possibility that deserves further investigation in the retina. In the skin we have a different situation. NAT-2 can acetylate both arylamines and arylalkylamines (like dopamine), which opens another aspect of whether NAT-2 can modify dopamine's biologic activity via the inactivation of dopamine. Although these ideas are speculative, they serve to outline future research, which hopefully will lead to a greater understanding of the physiologic role that these NAT activities may play.

In summary, we have identified two NAT activities, NAT-1 and NAT-2, with distinctly different specificity characteristics in hamster skin. NAT-2 deserves special attention due to its ability to acetylate both arylamines and arylalkylamines like serotonin and dopamine. These findings form a background for our future studies on potential synthesis of melatonin in the skin as well as further examination of N-acetyldopamine and its role in this tissue.

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